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ORIGINAL PAPER

Ovule rescue efficiency of *Gossypium hirsutum* × *G. arboreum* progeny from field-grown fruit is affected by media composition and antimicrobial compounds

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Abstract Upland cotton (*Gossypium hirsutum* L.) is reproductively isolated from *G. arboreum* L. via post-zygotic breeding barriers. Literature on somatic embryogenesis of cotton suggests a number of media modifications that might also prove useful for ovule rescue of interspecific crosses. Additionally, endogenous microbes are common in field grown cotton and these potential contaminants must be controlled if interspecific progeny are to be obtained via large-scale field crossing followed by ovule or embryo culture. This study compared nine tissue culture media and two antimicrobial overlay treatments in a factorial design. The overlay treatments were: a 2 ml overlay of 250 mg l^{-1} cefotaxime, 50 mg l^{-1} tetracycline HCl, 2.5 mg l^{-1} amphotericin B, and 50 mg l^{-1} benomyl applied when the ovules were plated, and no overlay. All of the media in the factorial also contained 250 mg l^{-1} cefotaxime. Crosses were made in a field at Stoneville, MS between the upland cultivar Delta-Pine 90 and the *G. arboreum* accession A_2-190. Antimicrobial compounds greatly improved the efficiency of obtaining interspecific cotton progenies from field-grown fruit. Germination was not affected by the overlay nor did overlay treatment interact with media. Media significantly affected germination. Of the media studied, the highest frequency of germination was observed for MSB with 1.9 g l^{-1} additional KNO_3. The addition of 0.5 g l^{-1} asparagine and 1 g l^{-1} glutamine did not affect the number of seedlings obtained. A filter paper growing surface or the addition of 0.5 mg l^{-1} NAA and 0.05 mg l^{-1} kinetin were disadvantageous.

Keywords Antibiotic · Benomyl · Cefotaxime · Cotton · Interspecific hybridization

Abbreviations

IAA	Indole acetic acid
MS	Murashige and Skoog (1962) medium
MSB	MS with B5 vitamins (Gamborg et al. 1968)
MSB2K	MSB with double the KNO_3 (+1.9 g l^{-1} additional)
NAA	α-Naphthaleneacetic acid

Introduction

Upland cotton, *Gossypium hirsutum* L., is a tetraploid (AADD genomic constitution) that originated in the Americas and is one of four independently domesticated *Gossypium* species (Brubaker et al. 1999). Upland cotton has become the predominant cotton species of commerce in the world primarily due to its high yield and the requirements of mechanized spinning for long

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fibers (May and Lege 1999). *G. arboreum* is a domesticated diploid (AA genomic constitution) Asian species that is a potential source of genes for upland cotton improvement, especially drought tolerance, and resistances to pests and diseases (Blank 1971; Gotmare and Singh 2004; Kapoor 2003; Knight 1948; Patil et al. 2003). However, upland cotton is reproductively isolated from *G. arboreum* L. via post-zygotic breeding barriers (Beasley 1940; Pundir 1972), in addition to the difference in chromosome number.

Ovule culture and embryo culture have been used to circumvent the breeding barrier between upland cotton and *G. arboreum*. Eid et al. (1973) found that upland cotton ovules grew better on MS than the media of White, Nitsch or Heller, and concluded that the relatively high nitrogen content of MS was advantageous. Given the importance of nitrogen for the development of cotton ovules into seedlings, Stewart and Hsu (1977) modified Beasley and Ting's (1973) cotton ovule media and found that increasing the NH_4^+ concentration up to 10–15 mM increased the number of mature and germinated upland cotton embryos obtained. Subsequently, Stewart and Hsu (1978) found that their media allowed them to recover *G. hirstum* × *G. arboreum* hybrids, and that the addition of IAA, kinetin and/or gibberellic acid did not improve recovery of mature and germinated embryos from cultured ovules. Gill and Bajaj (1987) found that supplementing MS with 250 mg l^{-1} casein hydrolysate, 1 mg l^{-1} IAA and 0.2 mg l^{-1} kinetin improved the recovery of germinated *G. hirsutum* and *G. hirstum* × *G. arboreum* seedlings from cultured ovules.

Efforts to develop somatic embryo culture systems for upland cotton have identified modifications of MS that might also be useful for improving the recovery of *G. hirstum* × *G. arboreum* seedlings from cultured ovules. Doubling the KNO_3 concentration of MS by adding an additional 1.9 g l^{-1} has been found to improve somatic embryo initiation and maturation (Davidonis and Hamilton 1983; Trolinder and Goodin 1988; Wu et al. 2004). Wu et al. (2004) found that adding asparagine (0.5–1.0 g l^{-1}) and glutamine (1.0–2.0 g l^{-1}) to MSB with double the KNO_3, significantly increased the number of somatic embryos that were converted into plants. Sakhanokho et al. (2001) found that somatic embryo development and maturation were improved by adding 0.5 mg l^{-1} NAA and 0.05 mg l^{-1} kinetin to MSB with double the KNO_3, 0.5 g l^{-1} asparagine, 1.0 g l^{-1} glutamine but lacking NH_4NO_3. Kumria et al. (2003) observed that a filter paper growing surface over solid MS media increased the number of globular somatic embryos that developed into plants relative to controls that were in direct contact with the media.

An additional consideration for obtaining interspecific cotton progeny is the need to make a sufficient number of crosses to ensure that at least one or more hybrid plants will be recovered. To make a sufficiently large number of crosses, it may be necessary to grow the parents in a field, where space is not as limiting as in a greenhouse. However, greenhouse-grown material typically has fewer potential microbial contaminants than field-grown material. Moreover, endogenous bacterial and fungal contaminants are common in cotton and its close relative, hibiscus (Adams and Kloepper 2002; Misaghi and Donndelinger 1990; Odutayo et al. 2004). Thus, potential exogenous and endogenous contaminants must be controlled if interspecific progeny are to be obtained via large-scale field crossing followed by ovule or embryo culture.

Methods to control endogenous contamination of plant tissue cultures typically rely on a mixture of antimicrobial compounds that have complementary modes of action. Successful strategies rely on combinations of antimicrobial compounds that are effective against the microbes yet non-toxic to the plant cultures (Pollock et al. 1983). Agrawal et al. (1998) found that the antibiotic cefotaxime typically promoted the growth of cultured cotton embryos. Antibiotics and antimycotics have been used on a variety of plant tissue cultures with varying success (Pence 2005; Thomas and Prakash 2004; Thurston et al. 1979). Little is known about how combinations of antibiotics and antimycotics might affect cotton ovule growth and development in vitro.

The main objective of this study was to compare media modifications based on earlier studies of cotton somatic embryogenesis and ovule rescue for effectiveness in obtaining *G. hirstum* × *G. arboreum* seedlings from cultured ovules. Additionally, the use of antimicrobial compounds for obtaining uncontaminated cultures from field-grown fruit was investigated.

Materials and methods

Nine tissue culture media and two antimicrobial overlay treatments were compared in a factorial design. The overlay treatments were: a 2 ml overlay

containing 250 mg l^{-1} cefotaxime, 50 mg l^{-1} tetracycline HCl, 2.5 mg l^{-1} amphotericin B and 50 mg l^{-1} benomyl applied when the ovules were plated, and no overlay. All of the media in the factorial also contained 250 mg l^{-1} cefotaxime. An additional treatment of one medium with no cefotaxime and no overlay was evaluated as a control.

During July and August 2005, crosses were made in a field at Stoneville, MS between the upland cultivar Deltapine 90 (PI 529529) and the *G. arboreum* accession A_2-190 (PI 615699). Seed of each line may be obtained from the National Plant Germplasm System (http://www.ars-grin.gov/npgs/). Fruits were harvested 5 d after pollination, washed with soap and water, then surface sterilized in a laminar flow hood by immersion in an aqueous solution of 2.6% sodium hypochlorite and 0.1% Tween-20 for 10 min with intermittent shaking by hand (inversion about every 5–10 s), followed by immersion in 100% ethanol for 10 min, and then allowed to air-dry. This surface sterilization protocol was previously found to be >99% effective on greenhouse-grown fruit (data not shown). The top and bottom ends of the fruit were removed aseptically with a scalpel. Four shallow longitudinal cuts were then made along the exterior wall of the fruit. To gain access to the ovules, the fruit was subsequently pulled apart into four sections using forceps and a scalpel. Individual ovules were separated from neighboring ovules using a scalpel. For each fruit, ovules were placed on a single 100 × 25 mm Petri dish containing 25 ml of media (Fig. 1). On average, a fruit contained 35 ± 6 ovules. Cultures were incubated at 30°C with 12 h of fluorescent light each day.

For the microbial contamination data, analyses of variance were performed with SAS procedures GLIMMIX and Fisher's Exact tests were performed with SAS procedure FREQ. Only Petri dishes that were free of visible microbial contamination were included in the germination analysis. For the germination count data, analyses of variance were performed with SAS procedures GLIMMIX; a Poisson response distribution was used and means in the original units were obtained. Media and overlay treatments were considered fixed effects.

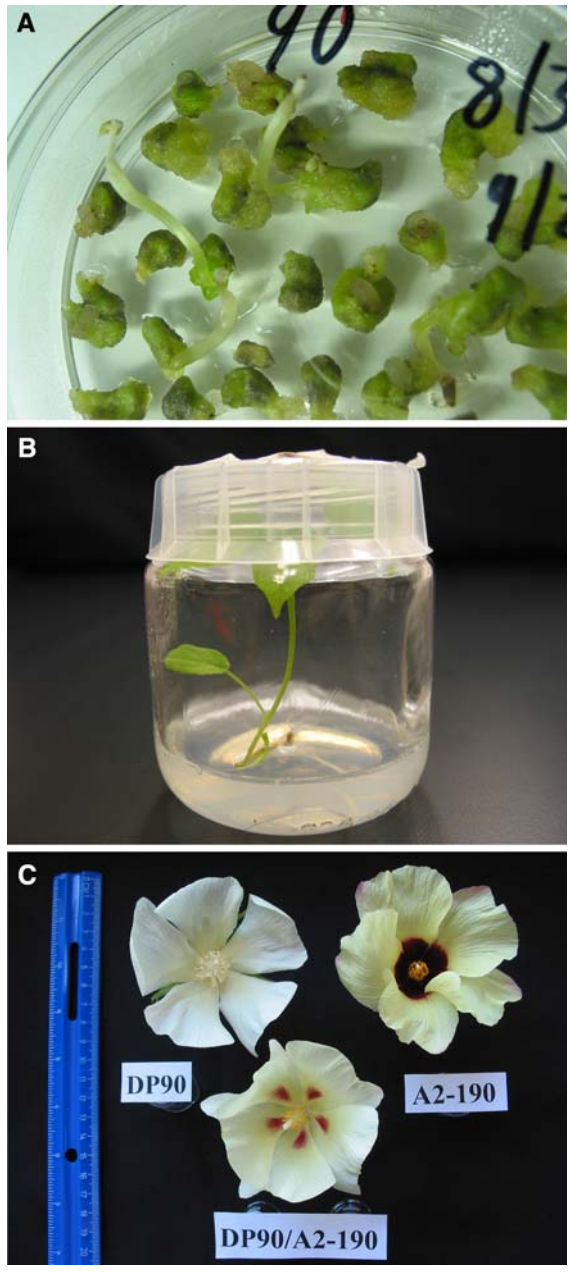

Fig. 1 Ovule rescue of *G. hirsutum* × *G. arboreum* (Deltapine 90 × A_2-190) cotton progenies: (**a**) seedlings germinating directly on media, (**b**) a seedling in vitro ready for transplanting to soil, (**c**) floral characteristics of the progenies were intermediate to those of the parents; note the cream petals and anthers of Deltapine 90, the yellow petals, bold purple petal-spots and yellow anthers of A_2-190, and the light yellow petals, moderate purple petal-spots and yellow anthers of the hybrid

Results and discussion

The addition of antimicrobial compounds had a highly significant effect on visible contamination frequency (Tables 1 and 2). All of the 11 non-antimicrobial control plates became contaminated (81.8% bacterial, 18.2% fungal). In contrast,

contamination was observed on only 62.1% of 169 plates with cefotaxime but no overlay (82.7% fungal, 10.6% both fungal and bacterial, 6.7% bacterial), and 5.6% of 54 plates with cefotaxime and an antimicrobial overlay (all bacterial). There were no significant differences in visible contamination frequency among media, nor interactions between media and overlay treatments (Table 1). Germination was not affected by the overlay nor did overlay treatment interact with media (Table 1). Thus, the use of antimicrobial compounds greatly improved the efficiency of obtaining interspecific cotton progenies when ovules from field-grown fruit were cultured.

Media significantly affected germination (Tables 1 and 2). Of the media studied, the highest frequency of germination was observed for MSB2K, which averaged seven seedlings per fruit, and this was significantly higher than the frequency observed for the MSB control (Table 2). Thus, doubling the KNO_3 concentration of MSB was highly advantageous.

Gamborg's B5 media (Gamborg et al. 1968) has had broad use for plant tissue culture and was effective for ovule rescue of interspecific tomato crosses (Sacks et al. 1997), *Lilium* crosses (Ikeda et al. 2003) and chicory crosses (Varotto et al. 2000). Since the concentration of KNO_3 and NH_4^+ in B5 media are considerably lower than those reported to be optimal for cotton in Stewart and Hsu's media (1977), we added additional KNO_3 and NH_4NO_3. However, the modified B5 media produced fewer seedlings than even the unmodified MSB (Table 2). Thus, other media components or interactions between other components with KNO_3 and NH_4^+ in B5 media likely hindered the development of interspecific cotton ovules.

The addition of 0.5 g l^{-1} asparagine and 1 g l^{-1} glutamine did not have a positive or negative effect on the number seedlings obtained (Table 2). Wu et al. (2004) found that double the concentration of asparagine and glutamine used in this study led to significantly more somatic embryos converted into plants, though they also found that the level used in this study was advantageous relative to a control lacking these amino acids. Further work will be needed to determine if greater concentrations of asparagine and glutamine would increase the frequency of recovering interspecific seedlings from ovule cultures.

A filter paper growing surface was disadvantageous for recovering interspecific cotton hybrids from cultured ovules (Table 2). Though Kumria et al. (2003) found that a filter paper growing surface increased the number of *G. hirsutum* somatic embryos that developed into plants, Sakhanokho et al. (2004) observed that, depending on the media, a filter paper growing surface either had no effect on or decreased the percentage of *G. arboreum* somatic embryos that germinated. The potential utility of a filter paper growing surface for cotton embryo and ovule cultures is doubtful.

Two media in this study included auxin (NAA or IAA) and the cytokinin kinetin at concentrations that were previously reported to be useful in cotton (Gill and Bajaj 1987; Sakhanokho et al. 2001) but these media produced fewer seedlings per fruit than MSB and MSB2K (Table 2). Stewart and Hsu (1978) also found that media without growth regulators produced more *G. hirstum* × *G. arboreum* seedlings than the many combinations of IAA and kinetin that they tested. The media named MSBCHH in Table 2 was very similar to the media described by Gill and Bajaj (1987), differing only in the substitution of 2% glucose for 3% sucrose to reduce browning, and gelrite for agar. However Gill and Bajaj (1987) observed no germination of *G. hirstum* × *G. arboreum* ovules without the growth regulators, and their results with the growth regulators were similar to those for the MSB2K media in this study. The different parental genotypes used in the two studies may account for the differing results obtained by the addition of auxin and kinetin. Little is known about the effect of genotype on the outcome of interspecific crosses in cotton, though in other crops parental genotype has been found to have a large effect on the relative difficulty of obtaining hybrids (Nimura et al. 2003; Sacks et al. 1997).

Table 1 ANOVAs comparing nine tissue culture media and two antimicrobial overlay treatments for endogenous contamination frequency of Petri dishes plated with *G. hirsutum* × *G. arboreum* ovules from field-grown fruit 5 days after pollination or number of seedlings that germinated per fruit (Petri dish) from aseptic cultures

Effect	DF	Contamination *P*	Germination *P*
Media	8	0.9076	<.0001
Overlay (OL)	1	<.0001	0.4017
Media × OL	8	0.6564	0.3983

Table 2 Frequency of microbial contamination and number of seedlings that germinated per fruit (Petri dish) from aseptic *G. hirsutum* × *G. arboreum* ovules cultured on different media

Media name	Basal medium	Additional components[c]	Contaminated petri dishes				Germinated seedlings		
			No overlay		Overlay[d]		Uncontaminated		
			No. observed	N[e]	No. observed	N[e]	Mean[f]	SE	N[e]
B5K50NH15	B5[a]	2.5 g l^{-1} KNO_3 + 1.04 g l^{-1} NH_4NO_3	10	17	0	5	1.8	0.4	12
B5K50NH15ag	B5	2.5 g l^{-1} KNO_3 + 1.04 g l^{-1} NH_4NO_3 + 0.5 g l^{-1} asparagine + 1.0 g l^{-1} glutamine	13	19	0	7	1.8	0.4	13
3/4MSBK50	¾ MSB[b]	3.63 g l^{-1} KNO_3	10	19	0	6	3.9	0.5	15
MSB	MSB	None	11	19	0	5	4.4	0.6	13
MSB2K	MSB	1.9 g l^{-1} KNO_3	12	20	1	6	7.0	0.8	13
MSB2Kag	MSB	1.9 g l^{-1} KNO_3 + 0.5 g l^{-1} asparagine + 1.0 g l^{-1} glutamine	12	23	0	7	6.7	0.6	18
MSB2KagFP	MSB	1.9 g l^{-1} KNO_3 + 0.5 g l^{-1} asparagine + 1.0 g l^{-1} glutamine + filter paper surface	12	15	0	7	2.2	0.5	10
MSB2KagH	MSB	1.9 g l^{-1} KNO_3 + 0.5 g l^{-1} asparagine + 1.0 g l^{-1} glutamine + 0.5 mg l^{-1} NAA + 0.05 mg l^{-1} kinetin	10	18	2	6	2.1	0.4	12
MSBCHH	MSB	0.25 g l^{-1} casein hydrolysate + 1 mg l^{-1} IAA + 0.2 mg l^{-1} kinetin	15	19	0	5	1.4	0.4	9

[a] Gamborg's B5

[b] Murashige and Skoog with Gamborg's B5 vitamins

[c] All media contained 20.0 g l^{-1} glucose and 2.2 g l^{-1} gelrite

[d] A 2 ml overlay containing 250 mg l^{-1} cefotaxime, 50 mg l^{-1} tetracycline HCl, 2.5 mg l^{-1} amphotericin B, and 50 mg l^{-1} benomyl applied when the ovules were plated

[e] Total number of plates (= number of fruit) studied

[f] Least square means from the model in Table 1, which included antimicrobial overlay treatments and interactions (all non-significant)

Seedlings from ovule culture that were grown to flowering size in a greenhouse were observed to have flower traits that were intermediate to those of the parents (Fig. 1). The progenies were also sterile, as expected for triploids. Some of the sterile triploid hybrids were subsequently treated with chromosome doubling compounds and fertile plants were recovered. Crossing these 2(AAD) hexaploids with D-genome diploid species will yield AADD tetraploids that have the same genomic constitution as upland cotton.

Thus, this study demonstrated that adding anti-bacterial and antifungal compounds could be highly advantageous for ovule rescue of interspecific cotton crosses derived from field-grown fruit. In addition, a simple modification of MS media (doubling the KNO_3) can improve the efficiency of recovering *G. hirsutum* × *G. arboreum* progeny. This study has also enabled us to obtain interspecfic hybrids that will be useful for cotton improvement. The *G. arboreum* accession used, A_2-190, is a source of resistance to reniform nematodes (Stewart and Robbins 1995). The 2(AAD) hexaploids will also be useful as bridging lines for introgressing genes from the D-genome diploid species into upland cotton.

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